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Irreversibly Sickled Cell β-Actin: Defective Filament Formation

Archil Shartava,[1,2] William Korn,[2] Arvind K. Shah,[2] and Steven R. Goodman[1,2]*

[1]Department of Structural and Cellular Biology, University of South Alabama College of Medicine, Mobile, Alabama
[2]USA Comprehensive Sickle Cell Center, University of South Alabama College of Medicine, Mobile, Alabama

It has been demonstrated that cysteine modification in irreversibly sickled cell β-actin slows down the remodeling of membrane skeletons [Shartava et al.: J Cell Biol 128:805–812, 1995]. This slow remodeling can be due to alterations in spectrin-actin binding and/or actin-actin interactions in irreversibly sickled cell (ISC) membrane skeletons. In these studies we demonstrate that ISC actin binds spectrin normally. However, ISC β-actin polymerizes and depolymerizes more slowly than control β-actin, and forms unusual aggregates when placed under polymerizing conditions. Electron microscopic analysis of actin polymers indicated that ISC actin generates a large amount of aggregates which we conclude are due to the structural modification caused by the disulfide bridge between cysteine^{284} and cysteine^{373} in β-actin. Am. J. Hematol. 55:97–103, 1997.

Key words: actin; spectrin; sickle-cell anemia

INTRODUCTION

Erythrocytes have a supramolecular organization of a two-dimensional latticework of structural proteins on the cytoplasmic surface of their membrane which supports the lipid bilayer and is essential in maintaining the biconcave shape and controlling elasticity of the cells in the high-shear environment of the vascular system [2]. The structural matrix of this two-dimensional meshwork, called the membrane skeleton, consists of spectrin, actin, and protein 4.1 (and other accessory proteins), and is linked to band 3 in the lipid bilayer through ankyrin [2,3]. Spectrin, the major protein of the skeleton, is primarily a tetrameric flexible rod of 200 nm in length [4]. Its tail regions are associated with actin protofilaments (a double-stranded helix with ~14 actin monomers), and this interaction is promoted by protein 4.1 [5–8]. Although it was proposed long ago that the formation of irreversibly sickled cells (ISCs) was caused in part by an alteration of membrane skeletal proteins [9], the mechanism of formation of ISCs was not well-elucidated.

Recently we demonstrated that a single posttranslational modification in ISC β-actin is a key determinant to the slow dissociation of the ISC membrane skeletal core proteins and the resultant slow remodeling of the ISC [1]. The modification is a disulfide bridge between cysteine^{284} and cysteine^{373} in β-actin which is not observed in control or reversibly sickled cells (RSCs) [1,10].

It has been previously demonstrated that the sickle-cell membrane has increased amounts of membrane proteins with oxidized thiols [11]. While some of the proteins (spectrin, band 3, and ankyrin) have reversible oxidation, others (band 4.1) are not reversible with reducing agents [12,13]. In recent studies we found that the β-actin oxidation in ISCs and the resulting slow remodeling of the ISC membrane skeletal proteins can be reversed with sufficient amounts of DTT [14]. Similarly, Ishiwata [15] indicated that cysteine oxidation of the actin molecule shifted the actin polymers to aggregates in reversible fashion when actin underwent one cycle of freezing and thawing. In this paper it is demonstrated that ISC actin substantially aggregates under polymerizing conditions and has a slower rate of polymerization and depolymerization than control actin. On the other hand, its ability to bind spectrin was not statistically distinct from that of control β-actin.

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*Correspondence to: Dr. Steven R. Goodman, Professor and Chairman, Department of Structural and Cellular Biology, University of South Alabama College of Medicine, MSB 2042, Mobile, AL 36688.

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MATERIALS AND METHODS

Isolation of Spectrin and Actin

All proteins were isolated as previously described [1]. For the isolation of ISC actin we used, in different experiments, blood from 6 individuals with homozygous SS sickle-cell anemia from 5 different families. Blood (40 ml from each subject) was obtained from 2 SS patients by venipuncture in vacutainer tubes containing 143 USP units of lithium heparin. Fresh blood was separated on the Percoll gradient, and the erythrocytes which migrated between layers 65% and 70% of the Percoll were used for further purification of the actin. As previously described, β-actin was isolated from a high ionic strength extract of core membrane skeletons by gel filtration chromatography on a Sepharose 4B column [1]. This procedure allowed us to purify ISC actin, which is monomeric and contains 0.2 thiols per molecule. Control spectrin and actin were isolated from 20 ml blood. At the final stage, spectrin was concentrated to 2–2.5 mg/ml in buffer containing 5 mM $NaPO_4$, 0.2 mM DTT, and 0.5 mM NaN_3, pH 7.8 (spectrin buffer). Control and ISC actins were concentrated to 0.4 mg/ml in 2 mM Tris, 0.2 mM ATP, and 0.5 mM NaN_3, pH 7.8 (actin buffer).

Spectrin-Actin Binding Assay

Equal concentrations of ISC or control actin were added to 0.25-ml centrifuge tubes (0.2 mg/ml final concentration). Five different concentrations of spectrin (from 0.07–1.1 mg/ml final concentration) were added to control and ISC actin. Samples were incubated at room temperature for 1 hr in a buffer containing 1 mM Tris, 2 mM $NaPO_4$, 50 mM KCl, 2 mM $MgCl_2$, 0.08 mM DTT, 0.3 mM ATP, and 0.4 mM NaN_3, pH 7.8, and then centrifuged for 1 hr at 100,000*g*. In control experiments, actin was omitted from the incubation mixture. Pellets were resuspended to the initial incubation volume in actin buffer and 10-μl aliquots were analyzed by SDS-PAGE. The SDS-PAGE was performed as described previously [1]. The amount of spectrin and actin was determined using laser densitometry vs. standards containing known concentrations of these proteins (Zeineh Soft Laser Densitometer, Biomed Instruments, Inc., Fullerton, CA). The amount of spectrin in the pellet minus the amount of spectrin sedimenting in the absence of actin was considered as bound, while spectrin in the supernatant was considered as free. The constant of dissociation (K_D) and maximal binding capacity (MBC) was calculated from nonlinear regression analysis of the binding curve and Scatchard analysis.

Actin Polymerization and Depolymerization Assay

Polymerization and depolymerization assays were carried out on a Hitachi F-2000 spectrafluorimeter following the increase or decrease in light scattering when excitation and emission wavelength were 450 nm. The rate of polymerization was computed using software package ''Enzfitter'' by Biosoft Co. (Cambridge, UK). The equation for the calculation was:

$$A = A_{max} (1 - e^{-kt}) + A_0$$

Where A is light scattering amplitude at time t, A_0 is an amplitude at time 0, A_{max} is amplitude at the end of measurement, and k is the polymerization rate constant. For the rate of depolymerization the following equation was used:

$$A = A_0 e^{-kt}$$

Monomeric control and ISC actin (0.36 mg/ml final concentration) were polymerized in a buffer containing 1.8 mM Tris, 2 mM $MgCl_2$, 50 mM KCl, 0.4 mM ATP, and 0.45 mM NaN_3 (final concentration), pH 7.8. Light scattering was continuously monitored for 1 hr at room temperature. For the depolymerization assay, polymerized actin was placed into buffer containing 0.6 M KCl,

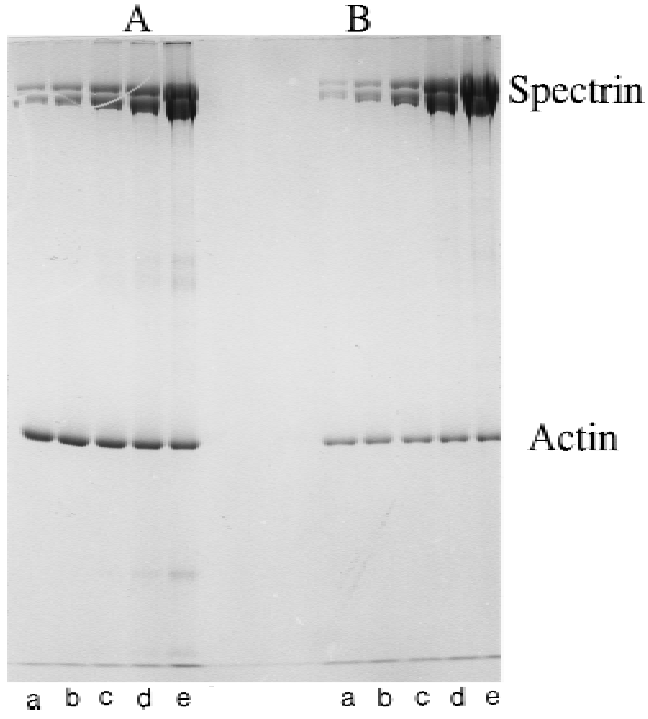

Fig. 1. SDS-PAGE of spectrin-actin binding assay. Various concentrations of spectrin (lane a, 0.072 mg/ml; lane b, 0.144 mg/ml; lane c, 0.289 mg/ml; lane d, 0.579 mg/ml; lane e, 1.158 mg/ml, final concentration) were added to equal concentrations (0.2 mg/ml) of ISC actin (A) and control actin (B). After polymerization of actin (1 hr at room temperature), bound and free spectrins were separated by centrifugation and pellets were analyzed by SDS-PAGE, as described in Materials and Methods.

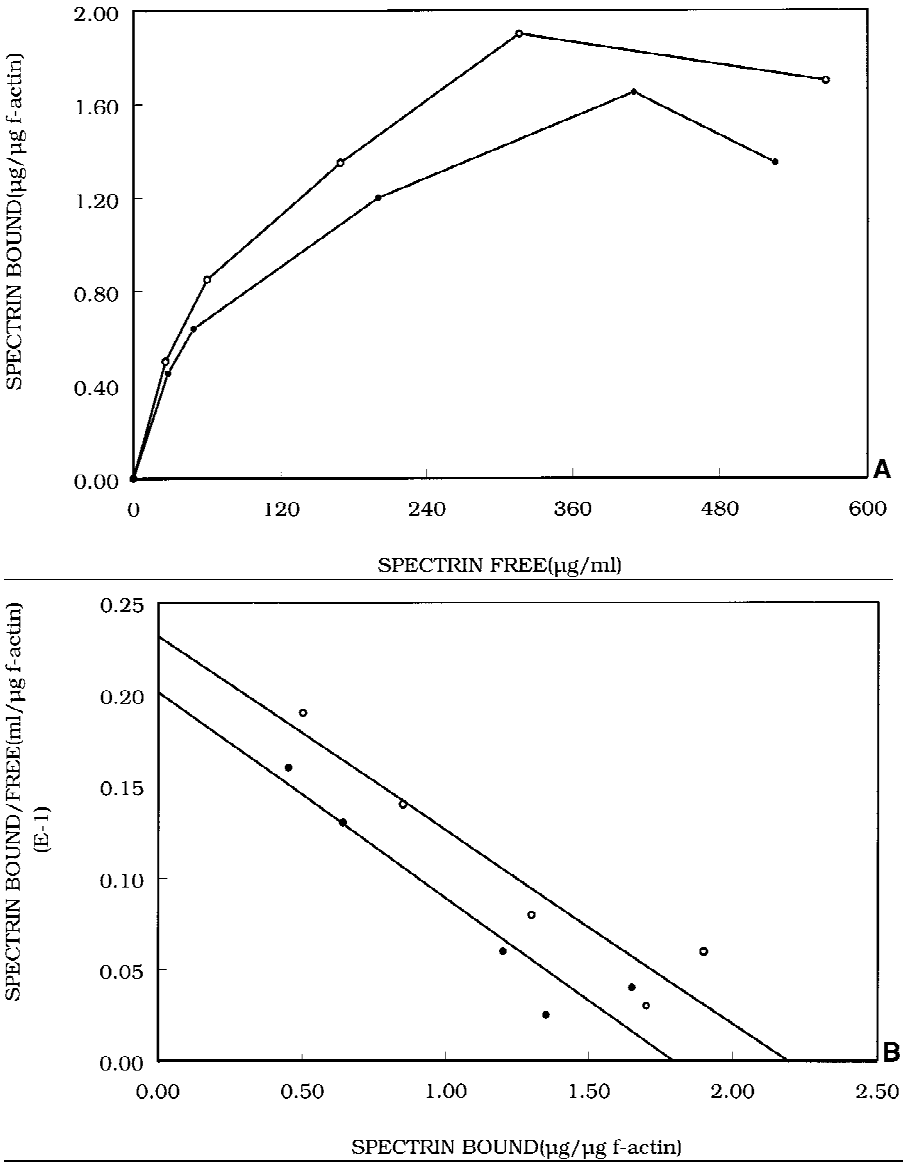

Fig. 2. Spectrin-actin binding isotherm. A: Various concentrations of spectrin were incubated with polymerized ISC and control actin, and bound and free spectrin were separated by centrifugation. Amounts of spectrin and actin were calculated using SDS-PAGE (Fig. 1) and laser densitometry. B: Data were plotted according to Scatchard analysis, and K_D and maximal binding capacity were computed. No statistically significant difference between ISC and control actin's ability to bind spectrin was found (-○-, control actin; -●-, ISC actin).

TABLE I. Spectrin-Actin Binding Analysis†

Protein	Maximum binding capacity (mg/mg)	K_D (nM)
Control actin	2.9 ± 0.8 (n = 3)	82.5 ± 23.3 (n = 3)
ISC actin	1.6 ± 0.4 (n = 3)	86.2 ± 43.3 (n = 3)
	P = 0.41	*P* = 0.95

†K_D and maximal binding capacity from spectrin-actin binding assay for ISC and control β-actin were statistically analyzed, and the summary of three independent experiments is presented here. The observed differences were not statistically significant. Values are presented as mean ± standard error.

1 mM Tris, 0.1 mM ATP, and 0.25 mM NaN_3, pH 7.8. Final concentration of actin was 0.2 mg/ml.

Electron Microscopy and Negative Staining

A drop of polymerized actin suspension (0.1 mg/ml) was placed on the formvar-carbon-coated nickel grid and then withdrawn after 30 sec. The samples were stained with a 1% aqueous solution of uranyl acetate for 30 sec. The stain was withdrawn and the samples were dried in the air at room temperature. Preparations were examined in a Philips 301 electron microscope using an accelerating voltage of 60 kv.

Statistical Analysis

The paired t-test was performed to compare the dissociation constant and maximal binding capacity of spectrin to actin for two groups: spectrin-control actin and spectrin-ISC actin. For polymerization and depolymerization assays the overall mean difference of the two groups (control actin and ISC actin) and corresponding standard error were computed and tested for significance, using a large sample test for contrast comparison.

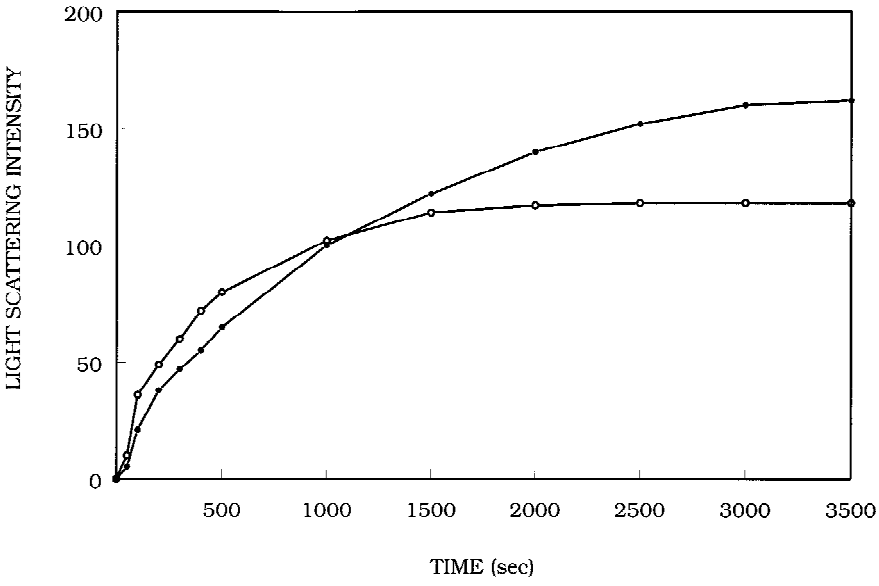

Fig. 3. Polymerization kinetics of ISC and control actin. We measured polymerization by continuous monitoring of an increase in 90° light scattering by spectrafluorimeter during a 1-hr incubation at room temperature. Polymerization was initiated by addition of polymerizing buffer into the cuvette containing monomeric ISC or control actin (0.36 mg/ml, final concentration) (-○-, control actin; -●-, ISC actin).

TABLE II. Actin Polymerization and Depolymerization Rates†

Protein	Polymerization light-scattering amplitude	Polymerization rate (10^{-3}) [S^{-1}]	Depolymerization rate (10^{-4}) [S^{-1}]
Control actin	133.2 ± 48.2* (n = 4)	1.6 ± 0.3* (n = 4)	3.54 ± 0.46* (n = 6)
ISC actin	160.5 ± 33.7 (n = 4)	1.1 ± 0.2 (n = 4)	2.32 ± 0.42 (n = 5)

†Under conditions described in Materials and Methods, actins from ISC and control RBCs were polymerized and depolymerized. Amount of polymerized actin was established from amplitude of light scattering, and rates of polymerization and depolymerization were computed using data obtained from the kinetics of these processes. Values are presented as mean ± standard error.

*Statistically significant difference between control and ISC actin ($P < 0.05$).

RESULTS

Spectrin-Actin Binding Assay

The aim of this assay was to identify if the molecular defect in ISC actin (SS-bond between cysteine^{373} and cysteine^{284}) can cause an alteration in the ability of actin to bind spectrin. We purified spectrin dimer and actin monomers from control RBCs and actin from ISCs. Increasing concentrations of spectrin were bound to polymerized control vs. ISC actin. Bound and free spectrins were separated and analyzed by SDS-PAGE and laser densitometry. A typical gel is represented in Figure 1. It is important to note that the amount of pelleted ISC actin is substantially higher than control actin. The quantity of bound spectrin was determined by laser densitometry, which was standardized with known quantities of pure spectrin loaded in separate wells for SDS-PAGE. Concentrations of free and bound spectrin were calculated, and K_D and MBC were estimated using nonlinear regression analysis and Scatchard analysis (Fig. 2) with little difference in the values.

A summary of three independent experiments is presented in Table I. There was not a statistically significant difference in K_D values (82.5 ± 23.3 vs. 86.2 ± 43.3 nM) or maximal binding capacity (2.9 ± 0.8 vs. 1.6 ± 0.4 μg/μg) for the interaction of control spectrin with control vs. ISC actin. Therefore, our next approach was to elucidate if the modification in ISC β-actin could cause a change in the actin-actin interaction.

Actin Polymerization and Depolymerization Assay

We used a 90° light-scattering technique for comparison of polymerization kinetics of control and ISC actins, which has been shown to be a useful assay for polymer weight concentration [16]. In a typical polymerization experiment (Fig. 3), light scattering was monitored for 1 hr. The summary of four different experiments (four different purifications of actin) is presented in Table II. Using a small sample test contrast comparison, we found that rate of polymerization of control actin was significantly higher than of ISC actin. Assuming that the size of the light-scattering amplitude is a measure of the amount of polymerized actin, ISC β-actin polymerizes with a lower rate constant, but demonstrates a significantly higher signal of light scattering at steady state (Fig. 3).

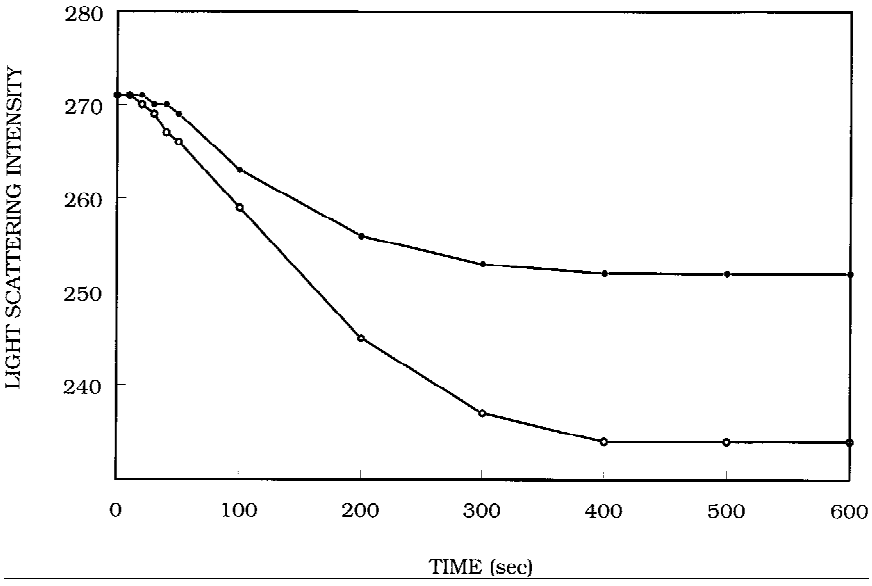

Fig. 4. Depolymerization kinetics of ISC and control actin. We measured depolymerization by monitoring of light scattering during 10 min at room temperature. Depolymerization was started by adding 0.6 M KCl (final concentration) into the cuvette containing ISC or control actin (0.2 mg/ml, final concentration), which was prepolymerized 1 hr at room temperature (-○-, control actin; -●-, ISC actin).

This indicates that a larger size or number of ISC actin polymers was generated. When filamentous actin was placed into the depolymerizing buffer, the rate of ISC actin depolymerization was lower compared to that of control actin (Table II). This unusual trend of G-F and F-G transitions of ISC actin led us to the next experiments, to visualize the difference between control and ISC actin polymers using negative staining and electron microscopy.

Negative Staining and Electron Microscopy

Control and ISC actins at a concentration of 0.1 mg/ml were polymerized at room temperature for 1 hr and then negatively stained on the grids. Electron micrographs of polymerized ISC actin demonstrate a large amount of aggregates (Fig. 5C,D) sometimes attached to short and curved filaments, or separated from the polymers. The size of aggregates varied from 10–100 nm and covered the entire field. On the other hand, control actin filaments appeared to be much longer and straighter, and the presence of aggregates was insignificantly low (Fig. 5A,B).

DISCUSSION

In this study we have demonstrated that the affinity of erythrocyte spectrin for ISC actin is similar to that for control actin. The stoichiometry of spectrin association with ISC actin had no significant difference from the stoichiometry of spectrin association with control actin, based on cosedimentation analysis. In our experiments, more ISC β-actin was sedimenting than control actin placed under identical polymerization conditions.

Electron microscopic analysis of ISC actin polymers demonstrated the presence of a large amount of aggregated actin as well as the presence of normally polymerized actin. We found a wide variety of sizes and shapes of these aggregates, which are located individually or in large clusters, or attached to normally polymerized F-actin molecules. Control β-actin filaments were well-defined 8-nm-diameter polymers with various lengths (up to several microns). In our kinetic experiments the rate of ISC actin polymerization was significantly lower than for control actin, but the extent (amplitude) of polymerization was increased. A similar phenomenon (decrease in rate but not in extent of polymerization) was described by Oosawa and Maruyawa [17] for rabbit skeletal muscle actin on prolonged incubation at room temperature. Drewes and Faulstich [18] determined that G-actin in the absence of excess ATP and divalent metal ions undergoes a very slow structural transition, accompanied by a decrease in polymerization rate.

In our studies we investigated β-actin which was structurally modified, the process occurring in the membrane skeleton proteins of irreversibly sickled cells. Unlike the damage which occurs in a frozen actin molecule (as a result of mechanical stress by frozen molecules of water) causing cysteine^{373} to form an intermolecular disulfide bond [15], the mechanical and oxidative stress on the actin molecule in the ISC leads to an intramolecular disulfide bond between cysteine^{373} and cysteine^{284} of at least 90% of the ISC β-actin molecules [1]. This intramolecular modification of ISC β-actin changes the morphology of actin polymers (Fig. 5) in a fashion similar to that of the intermolecular modification caused by a cycle of freezing and thawing of normal F-actin [15]. Functional alterations of ISC actin are similar to those which occurred during prolonged storage of actin at room temperature or in absence of excess ATP and divalent metal ions [17,18]. In this study, we demonstrate that the well-defined posttranslational modification in ISC β-actin [1]

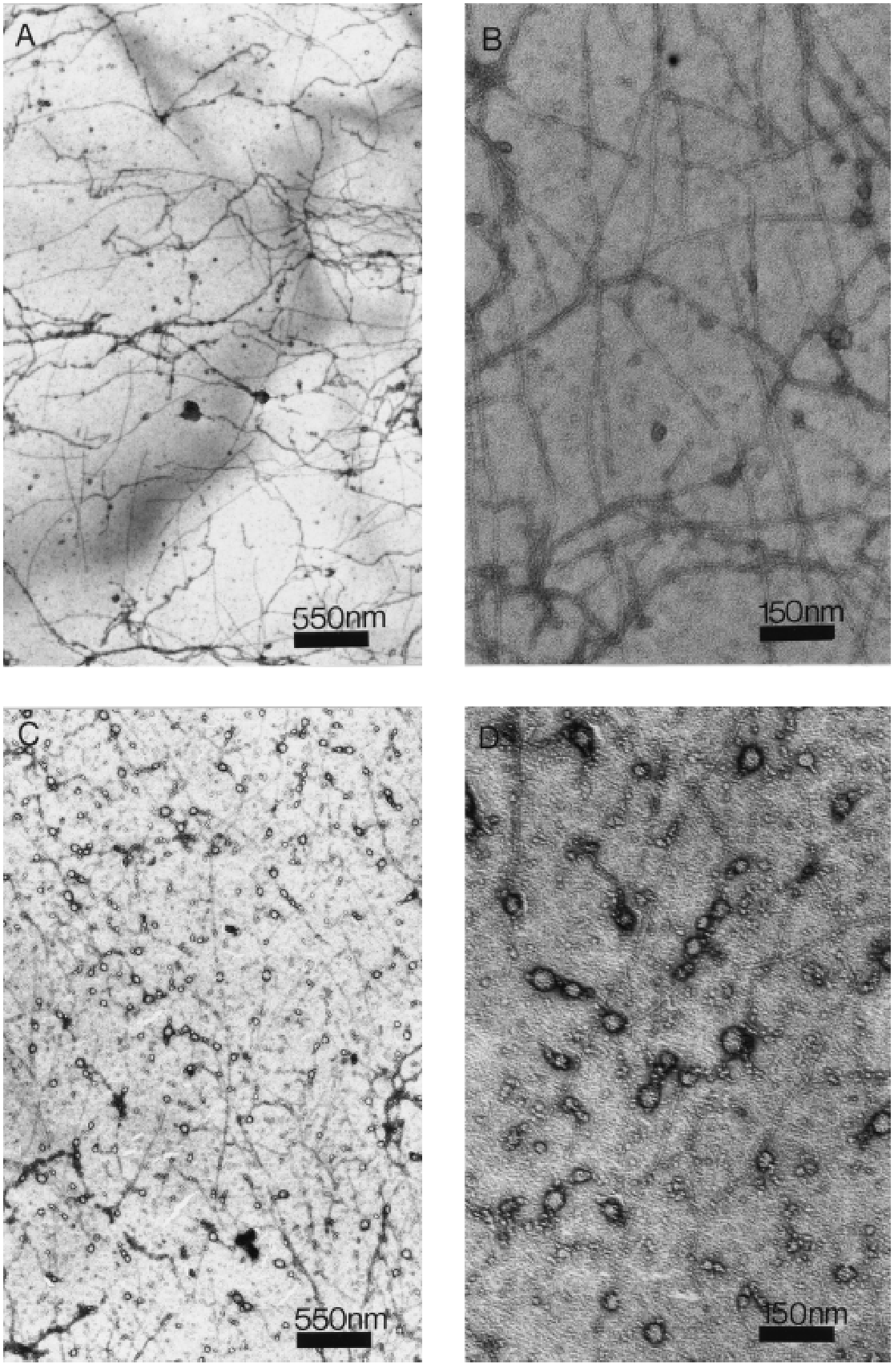

Fig. 5. Electron micrographs of ISC and control β-actins which were polymerized and negatively stained. A,B: High and low magnification of control F-actin. Actin appears to be normally structured, and different lengths of 80-nm-diameter filaments can be observed. C,D: Polymerized ISC actin appears to be mostly in aggregated form, partially associated with short and midsize actin filaments.

leads to an altered actin polymer formation, but does not effect the spectrin-actin interaction. The ISC actin is structurally altered in a manner previously demonstrated by our computer analysis of the three-dimensional structure of β-actin [1]. The aggregation of ISC β-actin is due to this structural modification and not to denaturation. Indeed, ISC β-actin is capable of polymerization, depolymerization, and normal binding of spectrin dimers. It will be of great interest to observe morphologic changes in actin filaments observed upon negative staining of spread ISC membrane skeletons.

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